

Studies on Components of Blood and its Functions

Chapter 3

Inflammation and Platelet Aggregation: The Role of Blood Extracellular Purines and Adenosine

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1. Introduction

Blood extracellular purines and adenosine, components of the purinergic system, are increasingly recognized as key mediators of activation and/or inhibition of circulating immune cells and platelets adhesion. This system is also composed by enzymes called ectonucleotidases and several receptors. In 1972, Burnstock introduced the concept of purinergic signaling [1]. After that, the role of purines and adenosine as signalling molecules has been studied in virtually all organic systems. Nucleotides- particularly ATP- are well known for their function as a universal energy currency. Interestingly, ATP has a completely different role in the extracellular compartment, where it binds to nucleotide receptors and initiate a signaling cascade. These receptors are called purinergic P2 receptors. In contrast to P1 receptors, which are activated by adenosine, P2 receptors are activated by ATP and/or other nucleotides (e.g.: ADP and UDP) [1,2].

On the basis of their signalling properties, P2 receptors can be further subdivided into metabotropic P2Y receptors (P2YRs) that are G-protein-coupled, and ionotropic P2X receptors (P2XRs) that are nucleotide-gated ion channels [3]. Although P2 receptors were originally described based on their functional role in the central nervous system, more recent studies demonstrate their widespread expression throughout different tissues and implicate them in innate or adaptive immune responses [4] as well as in platelet activation [5].

In this chapter, we will discuss the role of extracellular nucleotides and adenosine in

inflammation and in platelet aggregation, including: 1) the mechanisms by which ATP acts as a DAMP (damage associated molecular patterns) and adenosine acts as an anti-inflammatory molecule; and 2) the mechanisms by which ATP and ADP acts as a proaggregant molecules and adenosine acts as an antiaggregant molecule. We will further discuss the implication of these blood molecules in health and disease.

2. Purinergic System

The purinergic system plays important roles in the regulation of immune and platelet functions and do that at various levels [6,7]. The purinergic system encompasses (1) receptors that respond to extracellular purines, which are designated as P1 and P2 purinoceptors, (2) purine release and uptake, and (3) a cascade of enzymes that regulate the concentration of nucleotides and nucleosides near the cell surface. The P1 family has four subtypes of receptors: A1, A2A, A2B and A3. All of them are activated by adenosine [1,6]. The P2 receptors are divided into two major families: a ligand-gated ion channel P2X family and a G protein-coupled receptors P2Y family. There are seven ionotropic P2X receptors (P2X1-7), which are all activated by ATP, and eight P2Y receptor subtypes (P2Y1,2,4,6,11,12,13,14). From the latter metabotropic receptors, four of them respond to uracil nucleotides: P2Y2 and P2Y4 are activated by UTP, P2Y6 by UDP, and P2Y14 by UDP-glucose. P2Y2 is also activated as efficiently by ATP. P2Y11 receptor is activated preferentially by ATP, UTP being a less potent agonist. Interestingly, ATP is also an agonist at the rat P2Y4 receptor, but it may serve as a selective antagonist at the human P2Y4 receptor. Other P2Y receptors, namely P2Y1, P2Y12, P2Y13, are activated more potently by ADP than by ATP [1,6].

Since ATP and adenosine, as well as other nucleotides, play a role in immune system and platelet activation and have opposite effects, proper regulation of purinergic signaling by ectonucleotidases and adenosine deaminase (ADA) may be crucial to modulate immune cells and platelet functions [7,8]. Once released by a damaged tissue into the blood, ATP can be broken down by a cascade of ectonucleotidases into ADP, AMP and adenosine. By regulating the concentrations of extracellular nucleotides and nucleosides, especially ATP and adenosine, different combinations of ectonucleotidases would therefore be expected to have distinct effects on immune cells and platelet performance. Indeed, there are several ectonucleotidases and each of them differs in its biochemical properties, including substrate affinity/preference, formation of metabolites and optimal pH of activity. All immune cells and platelets express ectonucleotidases and ADA at different levels. The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) is a dominant family of ectonucleotidases that catalyzes the hydrolysis of nucleotides (Beaudoin et al., 1996; Knowles, 2011). It is composed of 8 members. The E-NTPDase members -1, -2, -3, and -8 (EC 3.6.1.5) are major enzymes responsible for the hydrolysis of nucleoside triphosphates and diphosphates at the cell surface under physiological conditions; NTPDase4, -5, -6 and -7 are mainly associated with intracellular organelles, such

as the Golgi complex. Isoforms -5 and -6 can be bound to the plasma membrane and released to the extracellular medium as soluble nucleotidases [1,8]. In the next sections the specific relationship between purinergic system and inflammation as well as the link between purinergic system and platelet activation will be discussed.

3. Purinergic System x Inflammation

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, and it is a response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, to clear out necrotic cells and damaged tissues from the original insult and to initiate tissue repair. Immune cells have developed highly sensitive receptor systems that allow them to execute their many roles in immune surveillance and host defense. To perform these tasks effectively, blood circulating neutrophils, for example, must detect trace amounts of the chemoattractants that help them locate and migrate to sites of infection and inflammation. Furthermore, immune cells must be stimulated to be able to proliferate and act as a body defenders. One of these stimulators are ATP, which is released in blood from damaged tissues associated with Inflammatory conditions. Also, stimulation of neutrophils and T cells leads to the rapid release of ATP, which triggers autocrine purinergic feedback loops that amplify the weak stimuli these cells receive during cell activation [3].

Not only ATP, but several purinergic signalling mechanisms regulate the activation of the different cell types of the immune system. For example, T cell activation induces the release of ATP through pannexin 1 channels that translocate with P2X receptors to the immune synapse with other immune cell, where they promote calcium influx and cell activation through autocrine purinergic signalling. Neutrophils release ATP in response to chemotactic mediators, and autocrine signalling through purinergic receptors regulates the chemotaxis of these cell. Activation of purinergic receptors in immune cells can elicit either positive or negative feedback mechanisms and thus can tightly regulate immune responses [3,9].

In addition to the autocrine feedback mechanisms that regulate the function of healthy immune cells, purinergic receptors allow immune cells to recognize the blood extracellular ATP that is released from damaged or stressed host cells. Thus, the purinergic signalling systems of immune cells acts in the recognition of 'danger' signals, and ATP which is released by stressed cells acts as a 'find-me signal' that guides phagocytes to inflammatory sites and promotes clearance of damaged and apoptotic cells. Purinergic signalling is also crucial for the activation of inflammasomes(responsible for activation of inflammatory processes) and the subsequent release of cytokines, such as interleukin-1 β (IL-1 β), in response to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) [9]. The purines released in the blood as danger signals from inflamed tissues as well as the role

of autocrine purinergic signalling systems in immune cell activation are crucial for inflammation [3].

Extracellular ATP is a danger signal released by dying and damaged cells, and it functions as an immunostimulatory signal that promotes inflammation. However, extracellular adenosine acts as an immunoregulatory signal that modulates the function of several cellular components of the adaptive and innate immune response. Consequently, the balance between ATP and adenosine concentration in blood is crucial in immune homeostasis [10].

3.1. Blood extracellular ATP act as a DAMP via P2 receptors

A main feature of a homeostatic system is the ability to rapidly sense any signs of distress that might signal the presence of exogenous or endogenous threat. This is even more important in the case of complex and integrated systems, such as living organisms that are characterized by a continuous exchange of information with the external world and at the same time undergo to a continuous process of internal self renewal (mitosis and apoptosis). Such a homeostatic system must be equipped with a fine network of sensors that constantly monitor the internal environment to detect even the most subtle signs of injury or distress [7,8]. Accordingly, a homeostatic system should be able to release an array of intracellular messengers capable of signaling cell and tissue damage. In a complex and integrated organism, the evolution has selected this signaling network that has no room for mistake, otherwise life would not thrive as we know [7].

One way to signal cell distress is through a receptor system that senses in the extracellular space the presence of molecules that are normally sequestered intracellularly. Therefore, it is not a surprise that the most powerful and ubiquitous signal of distress or damage (otherwise known as DAMP, damage-associated molecular pattern) is ATP. Of course, ATP is by no means the only DAMP used by multicellular organisms to signal danger, but it is likely to be the most ancient. In the healthy organism, ATP is almost exclusively present inside the cells, where it reaches millimolar concentration. In the extracellular environment the ATP concentration is negligible, i.e., low nanomolar range. The huge intra/extracellular chemical gradient, on the one hand, allows a fast release of ATP in response to the opening of plasma membrane ATP-conducting pathways. Furthermore, ATP is highly water soluble, and thus easily diffusible in the aqueous extracellular environment, and quickly degraded by several nucleotidases [1,11]. Last but not the least, virtually all eukaryotic cells are equipped with specific receptors for extracellular ATP, the P2 receptors. Thus, ATP is an ideal extracellular messenger of cellular distress and P2 receptors ideal sensors of danger [7].

Necrotic and apoptotic cells release ATP in blood, which can serve as a “find-me” signal that attracts monocytes to phagocytose and removal of dead or dying cells. This process involves the activation of P2Y2 receptors on monocytes, which are thought to follow ATP gradi-

ents to find their way to apoptotic cells. However, because of the short half-life of ATP in most tissues, it is uncertain whether this process alone could attract phagocytes over long distances. Recently, formylated peptides, which are released from the mitochondria of damaged cells, have been shown to induce neutrophil activation and chemotaxis in response to severe trauma and sterile inflammation. It is likely that such chemotactic mediators, together with chemokines and ATP, orchestrate the complex processes that guide phagocytes during their long-range approach and final encounter with target cells at inflammatory foci. This may also be true for pathological situations, such as those that follow severe trauma or cancer therapy, in which ATP release and danger signals emitted by damaged cells stimulate host immune responses through Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [4,6].

Interestingly, recent work has shown that extracellular ATP can promote NLR-mediated inflammasome assembly. Activation of the NOD-, LRR- and pyrin domain containing 3 (NLRP3; also known as NALP3) inflammasome has been shown to involve ATP release through pannexin 1 and purinergic signalling through P2X7 receptors. Inflammasome activation triggers innate immune defences by inducing the maturation of pro-inflammatory cytokines, such as IL-1 β , in a caspase 1-dependent manner. Activation of the NLRP3 inflammasome has also been observed in response to tumour cell destruction after cancer therapy. This process releases ATP and DAMPs, which stimulate P2X7 receptors and the NLRP3 inflammasome of dendritic cells, facilitating IL-1 β production and promoting immunity against tumours. The exact mechanisms that link purinergic signalling to NLRP3 inflammasome activation remain to be determined. However, it is intriguing to speculate that autocrine purinergic signalling events could be involved in this and other immune cell responses to DAMPs and PAMPs [3,10].

3.2. The role of adenosine as an anti-inflammatory molecule

Similar to ATP, it has been proposed that adenosine, the most important ATP degradation product, might also be an important danger signal. However, given its mainly immunosuppressive activity, this nucleoside is more likely to interfere at later stages of inflammatory process as an immunoregulatory feedback mediator [11,12]. Adenosine P1 receptors are widely expressed by immune cells of the myeloid and lymphoid lineage. The P1 purinoceptors, consist of 4 subtypes, A1, A2A, A2B and A3, all being G-protein coupled. The role of A1 and A3 receptors is not well understood, while there is some evidence for a crucial role of A2A and A2B receptors in the control of inflammation. In general, we can say that activation of A1 and A3 receptors seems to evoke pro-inflammatory effects, A2A and A2B receptors elicit anti-inflammatory effects [12].

The most important immunosuppressive mechanisms of adenosine signaling is its role in down-modulating multiple cytokine expression and secretion. Recent data have confirmed the role of adenosine in modulating chemokine secretion and chemokine receptor activation

and a deep investigation has been undertaken to shed light on the complex interplay between adenosine and chemokines in tuning leukocyte functions. Most of these studies indicate that adenosine through its receptors decreases chemokine production and dampens inflammation, thus reducing tissue damage. Hypoxic conditions induce adenosine formation in the extracellular milieu and adenosinergic receptors are involved in hypoxia-related signaling pathways. In particular, the A_{2A} subtype is involved not only in T cell apoptosis but also in the signaling pathway that reduces CCR7 expression under hypoxic conditions. The same subtype participates in the downregulation of CXCR4 and CCR5 induced by an agonist-like monoclonal antibody. A crucial aspect related to the establishment of hypoxic conditions is lack of infiltration of tumor mass by cytotoxic T lymphocytes. This is mainly due to stimulation by adenosine of the A_{2A} subtype [13,14].

4. Purinergic System x Platelet Aggregation

As seen in the previous section of this chapter, both ATP and their metabolites, especially adenosine, are deeply involved in the processes of hemostasis and inflammatory process. In this section we are going to review some aspects of ATP, ADP and adenosine in platelet activation. Platelets control bleeding (haemostasis) when there is an injury to the blood vessel wall, and the endothelial cell layer is disrupted exposing the adjacent extracellular matrix (EM). Then, platelets interact with different components of EM through adhesion receptors followed by a rapid signal transduction which leads to platelet activation, cytoskeletal changes associated with shape change, spreading and secretion, and inside–out activation of integrins that support adhesion and aggregation. In addition to this role for platelets in thrombus formation in normal haemostasis, essentially the same sequence of events results in thrombotic diseases such as heart attack and stroke [15,16]. Rupture of atherosclerotic plaque exposing the underlying fibrous matrix can lead to thrombus formation. Adhered activated platelets also interact with circulating leukocytes and facilitate platelet–leukocyte–endothelial cell adhesion. This involves receptors that also regulate thrombosis. Platelets promote the interaction of inflammatory leukocytes with the vessel wall in atherothrombosis, initiating development of atherosclerotic plaque that may eventually lead to thrombotic events [17,18].

4.1. Blood extracellular ATP and ADP are proagregant molecules

Platelet activation by adenosine diphosphate (ADP) is mediated by two purinergic G-protein coupled receptors: the P_{2Y}12 and P_{2Y}1 receptors. ATP may signal to platelets via P_{2X}1, a ligand-gated cation channel responsible for the fast influx of calcium [16].

P_{2Y}12 receptors are implicated in the potentiation of platelet aggregation and the reduction of intracellular cAMP production. The expression of the P_{2Y}12 receptor was long thought to be restricted to platelets and subregions of the brain. However, it has now been shown that this receptor is also expressed on vascular smooth muscle cells (VSMCs) and dendritic cells

(DCs). On blood platelets, the P2Y₁₂ receptor is entirely responsible for the role played by ADP in the amplification of aggregation, secretion, and stabilization of platelet aggregates and in enhancement of the procoagulant activity induced by agonists, such as thrombin, collagen, or thromboxane A₂. This signaling is not sufficient for the ADP-induced aggregation of platelets. For instance, the P2Y₁₂ receptor does not mediate platelet shape change, however, it potentiates the release of the contents from dense granules [17].

The central role of the P2Y₁₂ receptor in platelet activation and the growth and stabilization of a thrombus makes this receptor an attractive molecular target for antithrombotic agents (e.g.: in acute coronary syndromes). To highlight the importance of ADP signaling via P2Y₁₂ receptors, patients with severe P2Y₁₂ deficiency can experience serious hemorrhage [2,18]

In contrast to P2Y₁₂, P2Y₁ is widely distributed in many tissues, including blood vessels and blood cells, smooth muscle cells, neural tissue, and the heart, testis, prostate, and ovary. On blood platelets, ADP mediated activation of P2Y₁ triggers platelet shape change - a loss from their normal discoid shape - and aggregation, but weaker and more transient than P2Y₁₂ activation. Despite P2Y₁ wide expression, studies from platelet P2Y₁ receptor knock-out have shown a similar protective effect from thrombosis when compared to a whole body P2Y₁ $-/-$ mice. This may suggest that the platelet receptor is probably entirely responsible for the contribution of P2Y₁ signaling event that mediates thrombosis. To sum up, the relationship which best define these two platelet ADP receptors is that P2Y₁ initiates aggregation by changing platelet shape, other agonists (including collagen, thromboxane A₂ or thrombin) promotes an increase in ADP release from dense granules which acts on P2Y₁₂ receptors. This latter receptor is responsible for autocrine mechanism for enhancing all steps in promoting stable platelet aggregation [2,16].

4.2. Blood extracellular adenosine has antiaggregant properties

Adenosine, a regulatory metabolite, is generated in response to inflammation, hypoxia, or cellular injury and triggers a signaling cascade of several events, including increasing oxygen supply/ demand ratio, angiogenesis, and anti-inflammatory processes. Adenosine is also an important mediator of inhibition of platelet activation, and its effects are mediated via G-protein coupled adenosine receptors which increases intracellular cAMP levels, an inhibitor of platelet activation. Adenosine is released from cells or generated outside the cells, depending on ATP and eventually ADP and AMP extracellular metabolism. A compromised membrane integrity or pathology involving inflammation, ischemia, or hypoxia results in a significant increase in extracellular ATP, as seen previously in this chapter. Cells can be induced to release ATP through nerve stimulation, hypotonic stress, and mechanical stress. Upon release from cells, ATP is rapidly degraded by a series of membrane bound enzymes. Once adenosine is

generated, it can exert its effects through adenosine receptors A2A and A2B located in platelets membrane. Both are G protein-coupled hence which in the presence of adenosine they lead to the activation of adenylyl cyclase. Therefore, The A2A and A2B receptors inhibits platelet aggregation through elevation of intracellular cAMP. However, A2B seen to have a lower affinity to adenosine, suggesting the importance in adenosine concentrations and/or receptor density regulation [15,17].

An interesting relationship between the pro aggregant ADP receptor P2Y1 and the anti aggregant adenosine receptor A2B shows that receptor density plays an important role in platelets in thrombus formation. For instance, the absence of the A2B receptors leads to a consequent upregulation of the P2Y1 ADP receptor expression, while the presence of the A2B receptors and its activation leads to an elevation of cAMP and a down-regulation of the P2Y1 receptor. Thus, in addition to the A2B mediating direct effects by increasing cAMP levels, the receptor also downregulates the expression of an ADP receptor, further contributing to the control of platelet activation. Indeed, the expression of an active A2B receptor was found to be upregulated by stresses, such as oxidative stress, or vascular injury [2,16]. Thus, adenosine receptor activation is a likely contributor to elevated intracellular cAMP under stress.

5. Conclusion

Blood extracellular ATP and its metabolite adenosine are currently described as key mediators of the immune response and platelet activation. Extracellular ATP is a danger signal released by dying and damaged cells, and it functions as an immunostimulatory signal that promotes inflammation. However, extracellular adenosine acts as an immunoregulatory signal that modulates the function of several cellular components of the adaptive and innate immune response. In platelets, ADP is the main proaggregant molecule whereas adenosine has antiaggregant properties. Consequently, the balance between ATP and adenosine concentration is crucial in both immune and platelet homeostasis. Signals delivered by extracellular ATP and adenosine are detected and transduced by P2 and P1 receptors, respectively. Virtually all immune cells and platelets express P2 and P1 receptors, thus purinergic signaling affects all aspects of immunity and inflammation as well as of platelet aggregation. This realization has prompted a burst of novel investigations aimed at the design and synthesis of P2- or P1-targeted drugs for the treatment of chronic inflammatory diseases, cancer and diseases related to thrombus formation.

6. References

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